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(57) Abstract

The antimicrobial activity of albumin-type protein is described. Albumin-type proteins include 2S albumins, napins, barley trypsin inhibitor, wheat α-amylase inhibitor and cereal gliadins. The proteins exhibit a range of antifungal activity, and in addition can potentiate the antifungal activity of thionins. The proteins find commercial application as antifungal or antibacterial agents. Compositions containing an albumin-type protein, or an albumin-type protein and a thionin protein, may be applied externally to a plant, or may be expressed within a plant. Transformed plants will show increased disease resistance.

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BIOCIDAL PROTEINS

This invention relates to biocidal proteins and compositions, and processes for their manufacture and use. In particular, it relates to the antimicrobial activity of seed storage proteins, and to their synergistic activity with other antimicrobial proteins isolated from plants.

In this context, antimicrobial activity includes a range of antagonistic effects (such as partial inhibition or death) on a fungus and/or a bacterium. In particular, the invention relates to antifungal (which may include anti-yeast) activity.

Although plants normally grow on substrates that are extremely rich in fungal organisms, infection remains a rare event. To keep out potential invaders, plants produce a wide array of antifungal compounds, either in a constitutive or an inducible manner. The best studied of these are phytoalexins, secondary metabolites with a broad antimicrobial activity spectrum that are specifically synthesised upon perception of appropriate defence-related signal molecules. The production of phytoalexins depends on the transcriptional activation of a series of genes encoding enzymes of the phytoalexin biosynthetic pathway. During the last decade, however, it has become increasingly clear that some plant proteins can play a more direct role in the control of phytopathogenic fungi. Several classes of proteins with antifungal properties have now been identified, including β-1,3-glucanases (Mauch et al, 1988, Plant Physiol, 88, 936-942), ribosome-inactivating proteins (Roberts and Selitrennikoff, 1986, Biosci Rep, 6, 19-29; Leah et al, 1991, J Biol Ch m, 266, 1564-1573), chitinases

(Schlumbaum et al, 1986, Nature, 324, 363-367), chitin-binding lectins (Broekaert et al, 1989, Science, 245, 1100-1102) and permatins (Vig rs et al, 1991, Molec Plant-Microbe Interact, 4, 315-323; Woloshuk et al, 1991, Plant Cell, 3, 619-628).

The antifungal activity of thionins has also been reported (Apel et al, 1990, Physiol Plant, 80, 315-321). Thionins are basic cysteine-rich plant proteins that are thought to be involved in host defence (Rodriguez-Palenzuela et al, 1988, Gene, 70, 271-280).

The 2S albumins are, together with the 7S and 11S globulins, the main protein components in seeds of several different plant families, including the Brassicaceae (Higgins, 1984, Ann Rev Plant Physiol, 35, 191-221). They serve as storage molecules providing nitrogen and sulphur for the germinating seedling. The 2S albumins have been well studied in rapeseed (Brassica napus), where these proteins are termed napins. Napins are composed of two different chains of about 4 kDa (small subunit) and 9.5 kDa (large subunit), held together by two disulphide bridges. Both subunits originate from the same 21 kDa precursor polypeptide by post-translational proteolytic processing events (Crouch et al, 1983, J Mol Appl Genet, 2, 273-283; Ericson et al, 1986, J Biol Chem, 261, 14576-14581). Genes or cDNAs encoding napin precursors have been isolated from rapeseed (Scofield and Crouch, 1987, J Biol Chem, 262, 12202-12208; Josefsson et al, 1987, J Biol Chem, 262, 12196-12201), radish (Raphanus sativus) (Raynal et al, 1991, Gene, 99, 77-86) and Arabidopsis thaliana (Krebbers et al, 1988, Plant

Physiol, 87, 859-866). The 2S albumin genes belong to a large family of related g nes that are believed to have aris n by triplication of an ancestral protogene (Kr is and Shewry, 1989, Bio Essays, 10, 201-207). Other members of this superfamily include the barley (Hordeum vulgare) trypsin inhibitor, wheat α-amylase inhibitor, and cereal gliadins, all of which are considered as seed storage proteins. Unlike most 2S albumins, however, the aforementioned related proteins exist as uncleaved polypeptides in their mature form.

There has been speculation that seed storage proteins may have secondary functions related to the protection of seeds from damage caused by animals or micro-organisms. However, no such function has ever been attributed to 2S storage albumins.

We have now shown that 2S albumins and the related proteins (collectively described as "albumin-type proteins") exhibit surprising antifungal activity, and may be used as fungicidal agents. In addition, these proteins exhibit a surprising and unique property in that they can potentiate the antifungal activity of thionins: in some cases up to 70-fold.

According to the present invention, we provide an antimicrobial composition comprising an albumin-type protein. The said protein may be a functional polypeptide subunit of an oligomeric protein. The composition may contain more than one protein.

In further aspects, the invention provides a process of combating fungi or bacteria by exposure to such a composition.

The 2S albumins and related proteins show surprising activity: they inhibit the growth of a variety of plant pathogenic fungi. The antifungal properties of 2S albumins from rapeseed and radish and of a trypsin inhibitor from barley have been demonstrated. The antifungal activity of 2S albumins resides mainly in the small subunit of these proteins, and may be antagonised by K^+ and Ca^{2+} at physiological concentrations.

The albumin-type proteins (2S albumins and related proteins) show a wide range of antifungal activity, and could be used as fungicides by application to plant parts (or surrounding soil) using standard agricultural techniques (such as spraying). Pathogens exposed to the proteins are inhibited. The protein may eradicate a pathogen already established on the plant or the protein may protect the plant from future pathogen attack. The eradicant effect of the antimicrobial proteins is particularly advantageous.

The antimicrobial proteins can be extracted and purified from plant material, manufactured from their known amino acid sequence by chemical synthesis using a standard peptide synthesiser, or produced within a suitable organism by expression of recombinant DNA. DNA encoding the proteins can be manufactured using a standard nucleic acid synthesiser, or suitable probes (derived from the known sequence) can be used to isolate the actual gene(s) and control sequences from a plant genome. This genetic material can then be cloned into a biological system which allows expression of the proteins. Hence the proteins can be produced in a suitable micro-organism or cultured cell, extracted

and isolated for use. Suitable micro-organisms may include Escherichia coli and Saccharomyces cerevisiae. Suitable cells may include cultured insect cells and cultured mammalian cells.

The antimicrobial proteins may also be used to combat disease by expression of DNA encoding one or more of said proteins within transgenic plant bodies. The fungus or bacterium is thus exposed to the protein at the site of pathogen attack on the plant. In particular, by use of appropriate inducible gene regulator sequences, the albumins (seed proteins) may be produced in vivo within parts of the plant where they are not normally expressed in quantity but where disease resistance is important (such as in the leaves).

Plant cells may be transformed with recombinant DNA constructs according to a variety of known methods (Agrobacterium Ti plasmids, electroporation, microinjection, microprojectile gun, etc). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way, although the latter are usually more easy to regenerate. The progeny of these primary transformants will inherit the recombinant DNA encoding the antimicrobial protein(s).

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes,

carrot, lettuce, cabbage, onion.

The invention further provides a plant having improved resistance to a fungal or bacterial pathogen and containing recombinant DNA which expresses an albumin-type protein.

A pathogen may be any fungus or bacterium growing on, in or near the plant. In this context, improved resistance is defined as enhanced tolerance to a fungal or bacterial pathogen when compared to a wild-type plant. Resistance may vary from a slight increase in tolerance to the effects of the pathogen (where the pathogen in partially inhibited) to total resistance so that the plant is unaffected by the presence of pathogen (where the pathogen is severely inhibited or killed). An increased level of resistance against a particular pathogen or resistance against a wider spectrum of pathogens may both constitute an improvement in resistance.

Recombinant DNA is heterologous DNA which has been introduced into the plant. The recombinant DNA encodes an antimicrobial protein expressed for delivery to a site of pathogen attack (such as the leaves). The DNA may encode an active subunit of an antimicrobial protein, such as the small subunit (SS) of 2S albumin described in Example 10.

The invention further provides an antimicrobial composition comprising an albumin-type protein and a thionin protein. Either or both of said proteins may be a functional polypeptide subunit of an oligomeric protein. The composition may contain more than one albumin-type protein and/or more than one thionin protein.

In further aspects, the invention provides a

process of combating fungi or bacteria by exposure to such a composition.

Even more surprising than th ir own antifungal activity is the synergism exhibited between th albumins and the thionins. The unexpectedly potent antifungal activity of the albumin/thionin mixture can be exploited to combat plant disease. As above, the mixture may be used as a fungicide by application to plant parts (or surrounding soil) using standard agricultural techniques (such as spraying). Pathogens exposed to the proteins are inhibited. The protein may eradicate a pathogen already established on the plant or the protein may protect the plant from future pathogen attack. The eradicant effect of the antimicrobial proteins is particularly advantageous.

The antimicrobial proteins can be extracted and purified from plant material, manufactured from their known amino acid sequence by chemical synthesis using a standard peptide synthesiser, or produced within a suitable organism by expression of recombinant DNA as previously described.

The proteins may also be be used to combat disease by expression within transgenic plant bodies as previously described.

The invention further provides a plant having improved resistance to a fungal or bacterial pathogen and containing recombinant DNA which expresses an albumin-type protein and a thionin protein.

A plant with an inherent thionin content may be transformed with DNA encoding one or more albumin proteins, so that a mixture of the two classes of protein is produced in vivo.

Alternatively, DNA encoding one or more thionins can be transformed into a plant and expressed within tissue which has an inherent albumin content. A third possibility is to transform both DNA encoding one or more albumins and DNA encoding one or more thionins into a plant, so that both classes of protein are expressed together in the same tissue. The albumin-encoding DNA may be introduced on the same construct as the thionin-encoding DNA, or on a separate construct. Another possibility is to transform a first plant with DNA encoding an albumin-type protein and to transform a second plant with DNA encoding a thionin protein, and then to cross the first and second plants producing a plant containing recombinant DNA expressing both proteins.

The invention may be illustrated by the following examples and by reference to the drawings, in which:

Figure 1 is the chromatogram for the protein fraction extracted from radish seed.

Figure 2 is the chromatogram for the protein fraction extracted from rapeseed seed.

Figure 3 shows the SDS-PAGE analysis of the reduced and unreduced radish 2S albumins.

Figure 4 shows the SDS-PAGE analysis of the reduced and unreduced rapeseed 2S albumins.

Figure 5 shows the reversed-phase chromatogram of the reduced and carboxyamidomethylated Rs-2S5, a radish 2S albumin.

Figure 6 shows the amino acid sequences of the 2S albumins Rs-2S5, pBa3 and napin.

Figure 7 shows the N-terminal amino acid sequences of the trypsin inhibitors from barley and

wheat.

Figure 8 is a graph showing the d gree of Cercospora beticola disease on sugarbeet against rate of 2S albumin application.

Figure 9 is a graph showing the degree of Cercospora beticola disease on sugarbeet against time of 2S albumin application.

Figure 10 shows the nucleotide sequence of the 2S albumin gene pIG8.

Figure 11 shows the nucleotide sequence and derived amino acid sequence of the small subunit of 2S albumin fused to the Mj-AMP2 signal peptide.

Figure 12 shows the construction of the expression vector pIG13.

Figure 13 shows the construction of the expression vector pIG15.

Figure 14 shows the construction of the plant transformation vector pIG19.

Figure 15 shows the construction of the plant transformation vector pIG20.

EXAMPLE 1

Isolation of 2S albumins from radish and rapeseed seeds.

2S albumins were isolated from radish seeds and rapeseed seeds by a four-step procedure including ammonium sulphate fractionation, heat treatment, anion exchange chromatography and cation exchange chromatography. The detailed methods are described below.

One kg of radish or rapeseed seeds was ground in a coffee mill and the resulting meal was extracted for 2 hours at 4°C with 2 litres of an

ice-cold extraction buffer containing 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 2 mM EDTA, 2 mM thiourea and 1 mM PMSF. The homog nate was squeezed through cheesecloth and clarified by centrifugation (30 min at 7,000 x g). Solid ammonium sulphate was added to the supernatant to obtain 30% relative saturation and the precipitate formed after standing overnight at room temperature was removed by centrifugation (30 minutes at 7,000 x g). The supernatant was adjusted to 70% relative ammonium sulphate saturation and the precipitate formed overnight at room temperature collected by centrifugation (30 min at 7,000 x g). After redissolving the pellet in 400 ml H_2O the solution was heated at 80°C for 15 min. The coagulated insoluble material was removed by centrifugation (30 min at 7000 x g) and the supernatant was dialyzed extensively against distilled water using dialysis tubing (SpectralPor, Spectrum, USA) with a molecular weight cut off of 1000 Da. After dialysis the solution was adjusted to 50 mM Tris-HCl (pH 9) by addition of the ten-fold concentrated buffer, and subsequently passed over a Q-Sepharose Fast Flow (Pharmacia) column (12 x 5 cm) in equilibrium with 50 mM Tris-HCl, pH 9. The protein fraction passed through the column was dialyzed extensively against H_2O and adjusted to 50 mM sodium N-morpholinoethanesulfonic acid (MES)(pH6) by addition of the 10-fold concentrated buffer. About 150 mg protein of this fraction was applied on a S-Sepharose High Performance (Pharmacia) column (10 x 1.6 cm) previously equilibrated with the sodium MES buffer. The column was eluted at 2.5 ml/min with a linear

gradient of 1000 ml from 0 to 500 mM NaCl in 50 mM sodium MES buffer (pH 6). The eluate was monitor d for protein by online m asurement of th absorbance at 280 nm.

Figure 1 shows results for the radish seed extract and Figure 2 shows results for the rapeseed seed extract. The broad group of non-resolved peaks eluting between 250 and 450 mM NaCl (indicated by Numbers 1 to 5 in Figures 1 and 2) represent different isoforms of the 2S albumins. The protein fractions corresponding to these peaks are henceforward designated as Rs-2S1, Rs-2S2, Rs-2S3, Rs-2S4 and Rs-2S5, respectively, for the radish 2S albumins (Figure 1) and Bn-2S1, Bn-2S2, Bn-2S3, Bn-2S4 and Bn-2S5 respectively for the rapeseed 2S albumins (Figure 2).

EXAMPLE 2

Characterisation of the isolated 2S albumins.

The isolated isoforms of 2S albumins from radish and rapeseed were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) before and after reduction with dithiothreitol. SDS-PAGE was performed on precast commercial gels (PhastGel High Density from Pharmacia) using a PhastSystem (Pharmacia) electrophoresis apparatus. The sample buffer for analysis of unreduced proteins contained 200 mM Tris-HCl (pH 8.3), 1% (w/v) SDS, 1 mM EDTA, 0.005% bromophenol blue, and the sample buffer for analysis of reduced proteins contained a supplement of 1% (w/v) dithiothreitol (DTT). The unreduced proteins were fixed in 30% (v/v) ethanol/10% (v/v)

acetic acid and were silver-stain d according to Heukeshoven and Dernick (1985, Electrophor sis, 6, 103-112). Th reduced proteins were blotted on nitro-cellulose after electrophoresis and the blots were silver-stained according to Kovarik et al (1987, Folia Biologica, 33, 253-257).

Figure 3 shows the SDS-PAGE analysis of the radish 2S albumins in their unreduced (left panel) and reduced (right panel) forms. Lanes 1 show Rs-2S1, lanes 2 show Rs-2S2, lanes 3 show Rs-2S3, lanes 4 show Rs-2S4, lanes 5 show Rs-2S5. Figure 4 shows the SDS-PAGE analysis of the rapeseed 2S albumins in their unreduced (left panel) and reduced (right panel) forms. Lanes 1 show Bn-2S1, lanes 2 show Bn-2S2, lanes 3 show Bn-2S3, lanes 4 show Bn-2S4, lanes 5 show Bn-2S5. Fifty nanograms of the proteins were loaded on the gels. Lanes M show myoglobin fragments used as molecular weight markers (Pharmacia) with the following sizes: 17 kDa, 14.5 kDa, 8 kDa, 6 kDa, and 2.5 kDa.

As can be seen in Figure 3, the reduced radish 2S albumins migrate as 2 bands of about 9 and 4 kDa, respectively. In their unreduced form the different isoforms yield doublets with an apparent molecular weight of 17 and 18 kDa, respectively. In Rs-2S1 the 17 kDa band is predominant whereas in Rs-2S5 the 18 kDa band is the major component. The reduced rapeseed 2S albumins (Figure 4) migrate as 2 bands of about 9 and 4 kDa, respectively, whereas the unreduced 2S albumins migrate as single 17 kDa bands. The presence of the 4 and 9 kDa bands in the SDS-PAGE patterns of reduced radish and rapeseed 2S albumins is consistent with previous observations that these proteins contain a small (4

kDa) subunit and a large (9 kDa) subunit linked together by disulphide bridges (Crouch t al, 1983, J Mol Appl Genet, 2, 273-283; Laroche t al, 1984, Plant Physiol, 74, 487-493).

To confirm the identity of the isolated proteins as 2S albumins, one of the isoforms (Rs-2S5) was subjected to amino acid sequence analysis. Two hundred μg of Rs-2S5 was reduced with dithiothreitol and the cysteines were modified by S-carboxyamidomethylation as described by Creighton (1989, In: Protein structure, a practical approach, ed. Creighton T.E., pp. 155-167). The S-carboxyamidomethylated proteins were separated by reversed phase chromatography on a Pep S column (porous silica C2/C18, 25 x 0.4 cm, from Pharmacia). The column was eluted at 1 ml/min with a linear gradient (90 min) from 0 to 45% acetonitrile in 0.1% trifluoracetic acid.

Figure 5 shows the resulting chromatogram: the mixture separated into 2 groups of 3 peaks each. SDS-PAGE analysis of the different peaks revealed that peaks 1, 2 and 3 correspond to the small subunit (4 kDa) and peaks 4, 5 and 6 to the large subunit (9 kDa). The N-terminal sequence of material from peak 1 (small subunit) and peak 4 (large subunit) was determined by the Edman degradation method on a pulsed liquid-phase sequencer (Applied Biosystems model 477A/120). For the small subunit of Rs-2S5 (peak 1, Figure 5) 30 residues could be identified, whereas for the large subunit of Rs-2S5 (peak 4, Figure 5) 20 amino acids were sequenced.

The results of these analyses are shown in Figure 6, where the obtained sequences are compared

with those deduced from a radish 2S albumin cDNA clone, pBA3 (Raynal et al, 1991, Gene, 99, 77-86) and with data obtained by N-terminal sequencing of a rapeseed 2S albumin, napin (Ericson et al, 1986, J. Biol Chem, 261, 14576-14581). Amino acids differing from the corresponding residues in the Rs-2S5 sequences are indicated in lower case. The obtained partial sequence of the small subunit of Rs-2S5 is 100% identical to the pBA3 sequence and 87% identical to the napin sequence. The large subunit shows 90% identity with the corresponding pA3 and napin sequences.

EXAMPLE 3

Isolation of the trypsin inhibitors from barley seeds and α-purothionins from wheat seeds.

It is known from the literature that barley (Hordeum vulgare L.) contains a trypsin inhibitor that is highly homologous to 2S albumins from dicotyledonous species. However, in contrast to 2S albumins whose polypeptides are cleaved into a small and large subunit, the barley trypsin inhibitor (hereafter called bti0) consists of a single uncleaved 13 kDa polypeptide (Odani et al, 1983, Biochem J, 213:543-545).

In order to compare the antifungal activity of trypsin inhibitors to 2S albumins, bti0 was isolated by the procedure of Mikola and Suolinna (1969, Eur J Biochem, 9, 555-560). The preparation was further purified by an additional step consisting of reversed-phase chromatography on a Pep S (porous silica C2/C18) column (25 x 0.93 cm) (Pharmacia). The column was eluted at 5 ml/min

with a linear gradi nt (40 min) from 0 to 40% acetonitrile. The id ntity of the purified protein as bti0 was verified by N-terminal amino acid sequencing. The first 11 N-terminal residues were identical to those previously reported (Odani et al, 1983, J Biol Chem, 258, 7998-8000).

Moreover, by assaying the cation exchange chromatogram for trypsin inhibitor activity, a trypsin inhibitor fraction different from bti0 was discovered. This fraction was further purified by an additional step consisting of reversed-phase chromatography as described above. It yielded two peaks eluting at 33 and 35% acetonitrile, hereafter called bti1 (barley trypsin inhibitor 1) and bti2 (barley trypsin inhibitor 2) respectively. Amino-terminal sequence analysis revealed that both proteins are homologous to WG11, a Bowman-Birk type protease inhibitor from wheat (Odani et al, 1986, J Biochem, 100:975-983).

Bowman-Birk type trypsin inhibitors are known to occur also in barley endosperms (Boisen and Djertoft, 1982, J Sci Food Agric, 33:431-440) and some homology has been found between the Ricinis 2S albumin large subunit and the Bowman-Birk inhibitor from lima bean (Sharif and Li, 1982, J Biol Chem, 257:14753-14759).

Figure 7 shows the N-terminal amino acid sequences of the trypsin inhibitors from barley and from WG11.

α-Purothionin was isolated from wheat (Triticum aestivum) seed flour essentially as described by Redman and Fisher (1969, J Sci Food Agric, 20, 427-432). The preparation was purified by one more step consisting of reversed-phase

chromatography on a Pep S (porous silica C2/C18) column (25 x 0.93 cm) (Pharmacia). The column was elut d at 5 ml/min with a linear gradient (40 min) from 0 to 40% acetonitrile in 0.1% TFA. The purothionin eluted was two incompletely resolved peaks at 34 and 35% acetonitrile, respectively, which represent the α1 and α2 isoform (Mak and Jones, 1977, Cereal Chem, 54, 511-523). Both isoforms were pooled, to yield the α-purothionin preparation.

EXAMPLE 4

Antifungal activity assay.

Antifungal activity was measured by microspectrophotometry as previously described (Broekaert, 1990, FEMS Microbiol Lett, 69, 55-60). Routinely, tests were performed with 20μl of a (filter-sterilised) test solution and 80μl of a fungal spore suspension (2 x 10^4 spores/ml). The suspension was prepared either in growth medium A (half strength potato dextrose broth, Difco) or medium B (half strength potato dextrose broth, Difco, supplemented with 1 mM $CaCl_2$ and 50 mM KCl).

For experiments on the antagonistic effect of cations, a synthetic growth medium was used. The synthetic growth medium consisted of K_2HPO_4 (2.5 mM), $MgSO_4$ (50 μM), $CaCl_2$ (50 μM), $FeSO_4$ (5 μM), $CoCl_2$ (0.1 μM), $CuSo_4$ (0.1 μM), Na_2MoO_4 (2 μM), H_3BO_3 (0.5 μM), KI (0.1 μM), $ZnSO_4$ (0.5 μM), $MnSO_4$ (0.1 μM), glucose (10g/l), asparagine (1g/l), methionine (20 mg/l), myo-inositol (2 mg/l), biotin (0.2 mg/l), thiamine-HCl (1 mg/l), and pyridoxine-HCl (0.2 mg/l). Control microcultures

contain d 20 µl of steril distilled water and 80 µl of the fungal suspension.

Unless otherwise stated incubation was done at 25°C for 48 hours. Percent growth inhibition is defined as 100 times the ratio of the corrected absorbance of the control microculture minus the corrected absorbance of the test microculture over the corrected absorbance at 595 nm of the control microculture. The corrected absorbance values equal the absorbance at 595 nm of the culture measured after 48 hours minus the absorbance at 595 nm measured after 30 minutes.

EXAMPLE 5

Antifungal activity of the radish and rapeseed 2S albumins, barley trypsin inhibitors and α-purothionin.

The antifungal potency of the radish 2S albumins, rapeseed 2S albumins, barley trypsin inhibitors and α-purothionin was assessed by the microspectrophotometric assay described in Example 4. Growth of fungi, collection and harvest of spores was done as previously described (Broekaert et al, 1990, FEMS Microbiol Lett, 69, 55-60). Four different plant pathogenic fungi were used as test organisms: Fusarium culmorum IMI 180420, Alternaria brassicola MUCL 20297, Ascochyta pisi MUCL 30164, and Verticillium dahliae MUCL 19210.

Two different growth media were used in these tests: medium A was half-strength potato dextrose broth, and medium B was medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl. The constitution of monovalent and divalent cations of medium B is such

that it resembles that inside a plant c 11.

Serial dilutions of the different proteins were applied to the fungi, either in medium A or medium B. The concentration required for 50% growth inhibition after 48 hours of incubation (IC_{50} value) was calculated from the dose-response curves. The results of these experiments are summarised in Table 1. The following abbreviations are used for the fungi: Fc, Fusarium culmorum; Ab, Alternaria brassicola; Ap, Ascochyta pisi; Vd, Verticillium dahliae.

TABLE 1

ANTIFUNGAL ACTIVITY OF 2S ALBUMINS FROM RAPESEED AND RADISH, BARLEY TRYPSIN INHIBITORS AND α-PUROTHIONIN

Protein	IC_{50} (μg/ml)							
	Medium A				Medium B			
	Fc	Ab	Ap	Vd	Fc	Ab	Ap	
Rs-2S1	30	20	30	20	>1000	>1000	>1000	>1000
Rs-2S2	30	22	25	20	>1000	>1000	>1000	>1000
Rs-2S3	35	20	30	20	>1000	>1000	>1000	>1000
Rs-2S4	35	20	25	20	>1000	>1000	>1000	>1000
Rs-2S5	40	22	30	22	>1000	>1000	>1000	>1000
Bn-2S3	60	45	80	45	>1000	>1000	>1000	>1000
bti0	910	180	>1000	350	>1000	>1000	>1000	>1000
bti1	110	55	105	65	>1000	>1000	>1000	>1000
bti2	90	40	90	50	>1000	>1000	>1000	>1000
α-purothionin	4	4	8	2	11	11	12	8

In medium A (low ionic strength), the 2S albumins from rapeseed and radish, the barley trypsin inhibitors bti1 and bti2 and α-purothionin were active on all four fungi tested. The five isoform fractions of the radish 2S albumins had similar activities. The rapeseed 2S albumin and the barley trypsin inhibitors 1 and 2 had about two to three fold lower activities compared to the radish 2S albumins, whereas the thionins were about five to ten-fold more active relative to the radish 2S albumins.

In medium B (high ionic strength), however, the 2S albumins and the barley trypsin inhibitors were completely inactive at concentrations up to 1 mg/ml. In contrast the α-purothionin remained active in medium B, although its specific activity was decreased by about two- to four-fold in medium B relative to medium A.

EXAMPLE 6

Effect of ions on antifungal activity.

The effect of ions on the antifungal activity of radish 2S albumins was examined in more detail. The IC_{50} values of Rs-2S3 on Fusarium culmorum and Trichoderma hamatum MUCL 29736 were measured in five different media. The reference medium was the synthetic growth medium described in Example 4, which contains a total of 2.5 mM monovalent cations and 0.1 mM divalent cations. The four other media contained 10 mM KCl, 50 mM KCl, 1 mM $CaCl_2$ or 5 mM $CaCl_2$ in supplement, respectively.

Results are shown in Table 2.

TABLE 2

VARIATION IN ANTIFUNGAL ACTIVITY OF RS-2S3 IN PRESENCE OF K^+ AND CA^{2+}

Fungus	IC_{50} (μg/ml) Reference medium supplemented with: /	10mM K^+	50mM K^+	1mM Ca^{2+}	5mM Ca^{2+}
F culmorum	12	25	>400	>400	>400
T hamatum	3	3	12	100	>400

Addition of 50 mM K^+ to the medium decreased the antifungal activity of Rs-2S3 on F culmorum by more than 30-fold, while the activity on T hamatum was reduced by four fold. Also, the presence of Ca^{2+} at concentrations as low as 1 mM reduced the activity by more than 30-fold on F culmorum and by about 30-fold on T hamatum.

EXAMPLE 7

Synergism between 2S albumins, barley trypsin inhibitor and α-purothionin.

The synergistic antifungal effect of combinations of 2S albumins and α-purothionin, on the one hand, and barley trypsin inhibitors and α-purothionin, on the other hand, was measured as follows.

To serial dilutions of α-purothionin, a constant subinhibitory concentration of 2S albumins or barley trypsin inhibitors was added. The IC_{50} value of the α-purothionin was calculated from dose-response curves for the series with and without addition of 2S albumins or barley trypsin inhibitors. The subinhibitory concentrations of 2S albumins and barley trypsin inhibitors used in the experiment were 10 μg/ml (final concentration) for the tests in medium A and 10, 50 and 250 μg/ml for the tests performed in medium B. The Synergism Factor was calculated as the ratio of the IC_{50} value in the control series (no 2S albumins or trypsin inhibitors added) over the IC_{50} value in the series supplemented with 2S albumin or barley trypsin inhibitors. Synergism Factors are rounded to the nearest integer.

The results of these experiments are shown in Table 3. The following abbreviations are used for the fungi: Ab, Alternaria brassicola; Ap, Ascochyta pisi; Fc, Fusarium culmorum; Vd, Verticillium dahliae.

TABLE 3

SYNERGISTIC ANTIFUNGAL EFFECT OF COMBINATIONS BETWEEN 2S ALBUMINS/α-PUROTHIONIN AND TRYPSIN INHIBITORS/α-PUROTHIONIN

Fungus	Test Protein	IC_{50} of α-purothionin in μg/ml (Synergism Factor)					
		Medium A		Medium B			
		Test protein conc. (μg/ml)		Test protein conc. (μg/ml)			
		0	10	0	10	50	250
Ab	Rs-2S3	4	0.86(5)	11	6(2)	0.45(25)	0.25(44)
	Bn-2S3	4	0.22(18)	11	2(5)	0.32(34)	0.15(73)
	bti0	4	0.20(20)	11	2.5(4)	1.2(9)	0.35(31)
	bti1	4	0.8(5)	11	8(1)	2.5(4)	ND
	bti2	4	0.28(14)	11	8(1)	1.5(7)	0.2(55)
Ap	Rs-2S3	8	2.5(3)	12	5.5(2)	1(11)	0.8(14)
	Bn-2S3	8	2(4)	12	7(2)	3(4)	1.5(8)
	bti0	8	3(3)	12	10(1)	6(2)	1.2(10)
	bti1	8	7(1)	12	11(1)	9(1)	1.4(8)
	bti2	8	4(2)	12	12(1)	9(1)	1.2(10)
Fc	Rs-2S3	4	0.45(9)	11	3(4)	0.4(27)	0.6(18)
	Bn-2S3	4	0.25(16)	11	5(2)	1(11)	1.5(7)
	bti0	4	0.4(10)	11	10(1)	7(2)	0.9(12)
	bti1	4	1.5(3)	11	10(1)	2.5(4)	1.1(10)
	bti2	4	0.9(4)	11	10(1)	2.5(4)	1.2(9)
Vd	Rs-2S3	2	0.4(5)	8	1.1(7)	0.4(20)	0.4(20)
	Bn-2S3	2	0.15(13)	8	0.7(11)	0.22(36)	0.2(40)
	bti0	2	0.14(14)	8	2.8(3)	0.9(9)	0.4(20)
	bti1	2	0.6(3)	8	3(3)	0.9(9)	1.2(7)
	bti2	2	0.3(7)	8	1.5(5)	0.4(20)	0.8(10)

ND = Not determined.

With some exceptions, the synergism factors obtained in medium A were generally about two to three-fold higher than those obtain d in medium B. The highest synergism factor (73) was obtained in medium B for the combination of α-purothionin and Bn-2S3 (at 250 μg/ml) on the fungus Alternaria brassicola. The synergism factors increased drastically when the subinhibitory concentration of 2S albumins in medium B was raised from 10 to 50 μg/ml, but except for A brassicola, no further substantial increase was obtained on going from 50 to 250 μg/ml. With the barley trypsin inhibitor the synergism factors were generally between two and five-fold lower compared to the 2S albumins Rs-2S3 and Bn-2S3.

EXAMPLE 8

Effect of 2S albumins and α-purothionin on bacterial growth.

Antibacterial activity was measured microspectrophotometrically as follows. A bacterial suspension was prepared by inoculating either soft agarose medium C (10 g/l tryptone, 5 g/l Seaplaque agarose FMC), or soft agarose medium D (10 g/l tryptone, 5 g/l Seaplaque agarose (FMC), 1 mM $CaCl_2$ and 50 mM KCl). Aliquots (80 μl) of the bacterial suspension (10^5 colony forming units per ml) were added to filter-sterilised samples (20 μl) in flat-bottom 96-well microplates. The absorbance at 595 nm of the culture was measured with the aid of a microplate reader after 30 minutes (blank values) and 24 hours of incubation at 28°C.

Percent growth inhibition was calculated as described in Example 4 for th antifungal activity assay.

The following bacterial species were used: Agrobacterium tumefaciens LMG 188, Alcaligenes eutrophus LMG 1195, Azospirillum brasilense ATCC 29145, Bacillus megaterium ATCC 13632, Erwinia carotovora strain 3912, Escherichia coli strain HB101, Pseudomonas solanacearum LMG 2293, Sarcina lutea ATCC 9342. The antibacterial effect of Rs-2S3 was assessed by adding serial dilutions of the protein to bacterial suspensions. The highest test concentration was 500 µg/ml (final concentration).

Rs-2S3 only affected the growth of the gram-positive bacterium B megaterium (IC_{50} = 10µg/ml) and the gram-negative bacterium E carotovora (IC_{50} = 250µg/ml). However, when the growth medium C was supplemented with 1 mM $CaCl_2$ and 50 mM KCl (medium D), Rs-2S3 lost its inhibitory activity on these bacteria.

Synergisms in antibacterial activity were assessed for combinations between Rs-2S3 and α-purothionin by using the same approach as described in Example 7. A synergistic effect was observed on B megaterium. When tested in growth medium D, synergism factors of 4, 15 and 17 were obtained after addition of Rs-2S3 at subinhibitory concentrations of 10, 50 and 250 µg/ml, respectively, to a serial dilution series of α-purothionin (Table 4). Thus, the thionin-potentiating activity of 2S albumin is not limited to fungi, but is also evident on some bacterial species.

TABLE 4

SYNERGISTIC ANTIBACTERIAL EFFECT ON BACILLUS MEGATERIUM OF COMBINATIONS BETWEEN RS-2S3 AND α-PUROTHIONIN

Rs-2S3 conc. (μg/ml)	IC_{50} of α-purothionin in μg/ml (Synergism Factor)	
	Medium C	Medium D
0	2	2.5
5	0.6(3)	/
10	/	0.6(4)
50	/	0.2(15)
250	/	0.15(17)

EXAMPLE 9

Effect of 2S albumins and α-purothionin on cultured human cells.

Human cell toxicity assays were performed either on umbilical vein endothelial cells (Alessi et al, 1988, Eur J Biochem, 175, 531-540) or skin-muscle fibroblasts (Van Damme et al, 1987, Eur J Immunol, 17, 1-7) cultured in 96-well microplates. The growth medium was replaced by 80 μl of serum-free medium (Optimem 1 for endothelial cells or Eagle's minimal essential medium (EMEM) for fibroblasts, both from GIBCO), to which 20 μl of a filter-sterilised test solution was added. The cells were further incubated for 24 hours at

37°C under a 5% CO_2 atmosphere with 100% relative humidity. The viability of the cells was assessed microscopically after staining with trypane blue (400 mg/l in phosphate buffered saline, PBS) for 10 minutes. Alternatively, cells were stained with neutral red (56 ml/l in PBS) for 2 hours at 37°C. Neutral red treated cells were washed with PBS, lysed in acidic ethanol (30 mM sodium citrate, pH 4.2, containing 50% ethanol) and scored for release of the dye by microspectrophotometry at 540 nm.

The 2S albumins were evaluated for their potential toxic effects on mammalian cells. When added at up to 500 µg/ml to either cultured human umbilical vein endothelial cells or human skin-muscle fibroblasts, Rs-2S3 did not affect cell viability after 24 hours of incubation. In contrast, α-purothionin administered at 50 µg/ml and 20 µg/ml decreased the viability of both cell types by more than 90% and 50%, respectively. Addition of Rs-2S3 at a constant concentration of 250 µg/ml to a serial dilution series of a α-purothionin did not increase the toxic activity of the α-purothionin.

EXAMPLE 10

Antifungal activity of the small and large subunit of 2S albumins.

The small subunit (SS) and the large subunit (LS) of Rs-2S3 were prepared as follows. Rs-2S3 was reduced in 100 mM Tris-HCl, pH 8.4, 100 mM DTE and kept at 45°C for 1 hour. After reduction the preparation was passed over a reversed phase column as described in Example 2. The peaks corresponding to SS and LS, respectively, were pooled and

vacuum-dried. The cysteine residues of the SS and LS preparations were reoxidised using an oxido-shuffling syst m consisting of reduced glutathione and glutathione disulphide as described by Jaenicke and Rudolph (1989, In: Creighton (ed), Protein structure: a practical approach. IRL Press Ltd, Oxford, pp 191-224). After the reoxidation step the oxidised SS and LS were purified by reversed-phase chromatography. Their thiol content was assessed as described by Creighton (1989, In: Creighton (ed), Protein structure, a practical approach, pp 155-167) and found to be 9% and 22% of that of the reduced forms for SS and LS, respectively.

The antifungal activity of the reduced and re-oxidised SS and LS was determined on Fusarium culmorum as described in Example 4. The activity of the reduced forms was tested in the presence of subinhibitory concentrations of dithiotreitol (0.25 mM) to prevent spontaneous disulphide formation (Monsalve et al, 1991, Biochem Biophys Acta, 1078, 265-272). The reduced LS had no activity on F culmorum at concentrations below 400 μg/ml, while reduced SS had an IC_{50} value of 14 μg/ml. The oxidised SS had an IC_{50} value of 32 μg/ml, whereas oxidised LS had no activity below 400 μg/ml, when assayed in medium A. In medium B, the IC_{50} values of oxidised SS and LS were higher than 200 μg/ml and higher than 400 μg/ml, respectively.

The synergistic antifungal effect on F culmorum was assessed for combination between oxidised SS or LS and α-purothionin by using the same approach as described in Example 7. The results of these experiments are shown in Table 5.

TABLE 5

SYNERGISTIC ANTIFUNGAL EFFECT ON FUSARIUM CULMORUM OF COMBINATIONS BETWEEN OXIDISED SS OR LS AND α-PUROTHIONIN

Test protein	IC_{50} of α-purothionin, μg/ml (Synergism Factor)									
	Medium A			Medium B						
	Test protein conc. (μg/ml)			Test protein conc. (μg/ml)						
	0	10	20	0	10	20	50	100	500	
Oxidised SS	4	0.12(33)	ND	11	5.2(2)	ND	0.8(14)	ND	ND	
Oxidised LS	4	ND	0.32(13)	11	ND	6(2)	ND	0.9(12)	0.7(16)	

Table 5 shows that SS as well as LS (though to a much lesser extent) are able to potentiate the activity of α-purothionin. A synergism factor of up to 33 could be obtained when oxidised SS was added at 10 μg/ml to a dilution of α-purothionin and assayed in medium A. In medium B a synergism factor of 2 was measured under these conditions. A synergism factor of 14 was obtained when oxidised SS was added at 50 μg/ml in medium B. Low synergism factors were measured during the same experiments with the oxidised LS.

The antifungal activity and the thionin-potentiating activity of 2S albumins may apparently be attributed primarily to their small subunits. These results suggest that truncated 2S albumin genes encoding only the SS domain could be used for the expression of a novel type of antifungal protein. Since it has been shown that intramolecular disulphide bonds are formed spontaneously in purified reduced SS molecules (Monsalve et al, 1991, Biochem Biophys Acta, 1078, 265-272), the expressed proteins are likely to adapt the configuration necessary for exerting antifungal activity.

EXAMPLE 11

Anti-fungal activity of the 2S albumins against foliar disease: *in vivo* test

Radish 2S albumins were tested against the sugarbeet foliar disease *Cercospora beticola* (strain K897) to establish whether they exhibited any *in vivo* anti-fungal activity.

Sugarbeet plants wer grown in John Innes potting compost (No. 1 or 2) in 4cm diameter mini-pots. 27 day old plants w re used for th tests. The protein preparation was formulated immediately prior to use by dissolving in sterile distilled water and diluting to the appropriate concentration. The samples were applied to the plants as a foliar spray to maximum discrete droplet retention. The protein formulations were applied either one day prior to inoculation with the disease (1 day protectant assay) or 48 hours after disease inoculation (2 day eradicant assay). The pathogen was applied as a foliar spray at a concentration of 50,000 spores/ml. Following inoculation the plants were kept in a humidity chamber for 48 hours and the plants then moved to a 24°C growth room. Disease was assessed after a further 12 days.

Results are shown in Figure 8. When applied 2 days after inoculation of the disease (eradicant activity) the 2S albumins gave good disease control at rates of between 100 uM and 700 uM. Surprisingly though there was no disease control at rates of up to 700 uM when the protein was applied as a protectant 1 day prior to the disease.

To further investigate this difference the time at which the 2S albumins were applied relative to the disease was varied and the subsequent disease control assessed. The method was essentially as described above and the protein was applied at a single rate of 350 uM. The results are shown in Figure 9. Disease was applied at time zero and the proteins subsequently sprayed on at different times up to 96 hours after inoculation.

All the plants were assessed 14 days after inoculation with th disease. The level of disease on plants from each time point was compared to control plants treated in an identical way but sprayed with distilled water. It can be seen that maximum anti-fungal activity is obtained when the protein is applied between 24 and 72 hours after disease inoculation. It is likely that the plants own defence mechanism will also have been induced during this period and the expression of many AFPs including thionins have been shown to reach a maximum within 44 hours of pathogen attack (Bohlmann et al, 1988, EMBO J, 7:1559-1565). It is possible therefore that these in vivo results reflect the synergy demonstrated in vitro (example 7) and that the disease control that can be obtained by application of the 2S albumins results from the combination of the protein and a factor which is induced by the pathogen within the plant. This would account for the inability of the 2S albumins to give any disease control when applied at the same time as the disease or shortly afterwards. The reversal of disease control when the protein is applied 96 hours after inoculation probably reflects the fact that the fungus has become to well established.

EXAMPLE 12

Molecular cloning of the radish 2S albumin cDNA.

From outdoor grown Raphanus sativus plants, seeds at 6 different developmental stages were collected, frozen in liquid nitrogen and stored at

-80°C. After homogenisation with a mortar and pestle, total RNA was extracted from 15 g of a mixture of the 6 different developmental stages, using the method of De Vries et al (1988, Plant Molecular Biology Manual, B6, 1-13) with the exception that 6 ml of a 1:2 phenol:RNA extraction buffer mixture and 2 ml of chloroform were used per g of tissue. Poly $(A)^+$ mRNA was purified by affinity chromatography on oligo(dT)-cellulose as described by Siflow et al (1979, Biochemistry 18, 2725-2731) yielding about 10 µg of poly$(A)^+$ RNA per g of tissue. Double stranded cDNAs were prepared from 1,5 µg of poly$(A)^+$ RNA according to Gubler and Hoffman (1983, Gene 25, 263-269) and ligated to EcoRI/NotI adaptors using the cDNA Synthesis Kit of Pharmacia. The cDNAs were cloned into the lambda ZAPII phage vector (Stratagene) according to the manufacturers instructions. One of the radish 2S albumin cDNAs was cloned by performing PCR (polymerase chain reaction) (under standard conditions : Sambrook et al. 1989, Molecular cloning, Cold Spring Harbor Laboratory Press) on 2 µg of total cDNA as template DNA and using the sense oligonucleotide OWB18 (5'TAAGGATCCCATACACGAATGGCGAACAAGCTCTTCCTCG 3') and the antisense oligonucleotide OWB20 (5'TAATCTAGACTCTCGAGGGTTTCGTTTGG 3') as amplimers. OWB20 was designed based on the published sequence of the radish 2S albumin pBA3 cDNA (Raynal et al, 1991, Gene 99, 77-86). As this clone was truncated at its 5' end, OWB18 was designed based on the published sequence of the oilseed rape napin pNAP1 cDNA (Ericson et al, 1986, J. Biol. Chem. 261, 14576-14581). OWB18 has the TAAGGATCC ('TAA'

followed by th BamHI recognition site) at its 5' end and OWB20 the TAATCTAGA ('TAA' followed by the XbaI recognition site). The PCR-product was cut with BamHI and XbaI, subcloned into pEMBL18+ pre-digested with the same enzymes and subjected to automated nucleotide sequencing on a Pharmacia ALF Automated Nucleotide Sequencer. The nucleotide sequence of this clone (hereafter called pIG8) is given in Figure 10 (the signal peptide is underlined with a dashed line, the mature small subunit is underlined with a full line, the mature large subunit is boxed).

EXAMPLE 13

Molecular cloning of the radish 2S albumin small subunit cDNA and construction of pIG11

To obtain the 2S albumin small subunit cDNA, the same approach was followed as the one outlined in Example 12 though now using the sense oligonucleotide OWB19 (5'TAATCTAGACTAACCGGACTGCCTTGCCTGCC 3') and the antisense oligonucleotide OWB21 (5'AATTGCTAGCGCCGGCCCATTCAGGATTCC 3'). OWB19 carries the TAATCTAGACTA sequence ('TAA' followed by the XbaI recognition site and 'CTA' which introduces the stop codon 'TAG') at its 5' end and OWB21 carries at its 5' end the AATTGCTAGC sequence ('AATT' followed by the NheI recognition site). The Mirabilis jalapa antimicrobial protein 2 (Mj-AMP2 ; Cammue et al, 1992, J. Biol. Chem. 267, 2228-2233) signal peptide was obtained in the same way as described above though using 50 ng of the Mj-AMP2 cDNA clone pMJ9 as template DNA and the M13

universal primer and the antisense oligonucleotide OWB22 (5'AATTCTAGAATAGCTAGCTTCTATCATGCCTGACATGG 3') as amplimers. OWB22 carries the AATTCTAGAATAGCTAGC sequence ('AAT'-XbaI recognition site-'ATA'-NheI recognition site) at its 5' end. The Mj-AMP2 signal peptide PCR-product was cut with BamHI (occurring in the polylinker of pMJ9) and XbaI and cloned into pEMBL18+ pre-digested with the same enzymes. The additional ATG start-codon upstream of the Mj-AMP2 start-codon was removed by cutting the obtained construct with EcoRV and SmaI followed by blunt ligation. This resulted in the clone pIG10.

Subsequently, the radish 2S albumin small subunit PCR-product was cut with NheI and XbaI and cloned into pIG10 pre-digested with NheI. As NheI and XbaI produce compatible ends, the orientation of the inserted small subunit was checked by digestion of different clones with NheI and BamHI. One of the clones with the correctly inserted small subunit, pIG11, was subjected to automated nucleotide sequence analysis. The nucleotide sequence and the derived amino acid sequence of the pIG11 insert are given in Figure 11. Note that the first amino acid of the small subunit (a proline, see Fig. 10) has been changed into a serine. As the oilseed rape napins have a serine as the first amino acid of their small subunit (Ericson et al, 1983, J. Biol. Chem. 261, 14576-14581) and also exert antifungal activity and display the synergistic effect with the α-purothionin (see Examples 5 and 10), this substitution is not believed to affect nor the antifungal activity nor the synergistic effect of this altered radish 2S

albumin small subunit.

EXAMPLE 14

Construction of the expression vectors pIG13 and pIG15.

The expression vector pIG13 (Figure 12) contains the full coding region of the Rs-2S albumin cDNA flanked at its 5' end by the strong constitutive promoter of the 35S RNA of the cauliflower mosaic virus (Odell et al, 1985, Nature 313, 810-812) with a duplicated enhancer element to allow for high transcriptional activity (Kay et al, 1987, Science 236, 1299-1302). The coding region of the Rs-2S albumin cDNA is flanked at its 3' end side by the polyadenylation sequence of 35S RNA of the cauliflower mosaic virus (CaMV35S). The plasmid backbone of this vector is the phagemid pUC120 (Vieira and Messing 1987, Methods Enzymol. 153, 3-11). pIG13 was constructed as follows : clone pIG8 which consisted of the Rs-2S albumin cDNA (Figure 10) cloned into the KpnI / PstI sites of pEMBL18+ (Boehringer). The 298 bp KpnI / PstI fragment was subcloned into the expression vector pFAJ3002 which was pre-digested with KpnI and PstI. pFAJ3002 is a derivative of the expression vector pFF19 (Timmermans et al. 1990, J. Biotechnol. 14, 333-344) of which the unique EcoRI site is replaced by a HindIII site. The expression vector pIG15 (Figure 13) contains the hybrid nucleotide sequence coding for the Mj-AMP2 signal peptide followed by the Rs-2S albumin small subunit and was constructed in the same way as pIG13 though starting from pIG11 (Figure 11).

EXAMPLE 15

Construction of the plant transformation vector pIG19 and pIG20.

The expression vector pIG13 was digested with HindIII and the fragment containing the Rs-2S albumin cDNA expression cassette was subcloned into the unique HindIII site of pBin19Ri. pBin19Ri is a modified version of the plant transformation vector pBin19 (Bevan 1984, Nucleic Acids Research 12, 8711-8721) wherein the unique EcoRI and HindIII sites are switched and the defective nptII expression cassette (Yenofsky et al, 1990, Proc. Natl. Acad. Sci. USA 87:3435-3439) is introduced. The new plant transformation vector is designated pIG19 (Figure 14). The new plant transformation vector pIG20 was constructed in the same way as pIG19 with the exception that the HindIII fragment of pIG15 (containing the hybrid Mj-AMP2 signal peptide / Rs-2S albumin small subunit expression cassette) was subcloned in pBin19Ri (Figure 15).

EXAMPLE 16

Plant Transformation.

The disarmed Agrobacterium tumefaciens strain LBA4404 (pAL4404)(Hoekema et al, 1983, Nature 303, 179-180) was transformed with the vectors pIG19 or pIG20 using the method of de Framond et al, (BioTechnology 1, 262-269).

Tobacco transformation was carried out using leaf discs of Nicotiana tabacum Samsun based on the method of Horsch et al, (1985, Science 227, 1229-1231) and co-culturing with Agrobacterium strains containing pIG19 or pIG20. Co-cultivation was

carried out under selection pressure of 100 μg/ml kanamycin. Transgenic plants (transformed with pIG19 or pIG20) were regenerated on media containing 100 μg/ml kanamycin. These transgenic plants may be analysed for expression of the newly introduced genes using standard western blotting techniques. Plants capable of constitutive expression of the introduced genes may be selected and self-pollinated to give seed. F1 seedlings of the transgenic plants may be further analysed. Ultimately, transgenic homozygous (in the pIG19 or in the pIG20 trait) plants may be crossed with transgenic thionin-homozygous tobacco plants. The new transgenic plants may be analysed as above.

CLAIMS

1 An antimicrobial composition comprising one or more albumin-type proteins.

2 An antimicrobial composition as claimed in claim 1 and containing one or more thionin proteins.

3 A composition as claimed in claim 1 or claim 2 in which at least one protein is a functional polypeptide subunit.

4 A composition as claimed in any of claims 1 to 3 in which at least one albumin-type protein is a 2S albumin or a napin.

5 A composition as claimed in claim 4 in which at least one albumin-type protein is the small subunit of 2S albumin.

6 A composition as claimed in any of claims 1 to 5 in which at least one albumin-type protein is barley trypsin inhibitor, wheat α-amylase inhibitor, or a cereal gliadin.

7 A composition as claimed in any of claims 1 to 6 which is antifungal.

8 A process of combating fungi or bacteria which comprises exposing them to a composition as claimed in any of claims 1 to 7.

9 A process as claimed in claim 8 in which the composition is applied externally to a plant.

10 A process as claimed in claim 8 in which the composition is expressed within a plant.

11 A plant having improved resistance to a fungal or bacterial pathogen and containing recombinant DNA which expresses an albumin-type protein for delivery to a site of pathogen attack.

12 A plant as claimed in claim 11 in which recombinant DNA expresses a thionin protein for delivery to a site of pathogen attack.

13 A biological system having improved resistance to a bacterial or fungal pathogen, and expressing first and second proteins for delivery to a site of pathogen attack, said first protein being an albumin-type protein and said second protein being a thionin, characterised in that at least one of said first and second proteins is expressed from recombinant DNA.

14 A plant as claimed in any of claims 11 to 13 where the site of pathogen attack is in a leaf.

15 A plant as claimed in any of claims 11 to 14 in which at least one protein is a functional polypeptide subunit.

16 A plant as claimed in any of claims 11 to 15 in which at least one albumin-type protein is a 2S albumin or a napin.

17 A plant as claimed in claim 16 which contains recombinant DNA having substantially th sequence shown in Figure 10.

18 A plant as claimed in claim 16 in which at least one albumin-type protein is the small subunit of 2S albumin.

19 A plant as claimed in claim 18 which contains recombinant DNA having substantially the sequence shown in Figure 11.

20 A plant as claimed in any of claims 11 to 19 in which at least one albumin-type protein is barley trypsin inhibitor, wheat α-amylase inhibitor, or a cereal gliadin.

21 Seeds and progeny of a plant as claimed in any of claims 11 to 20.

FIG. 1

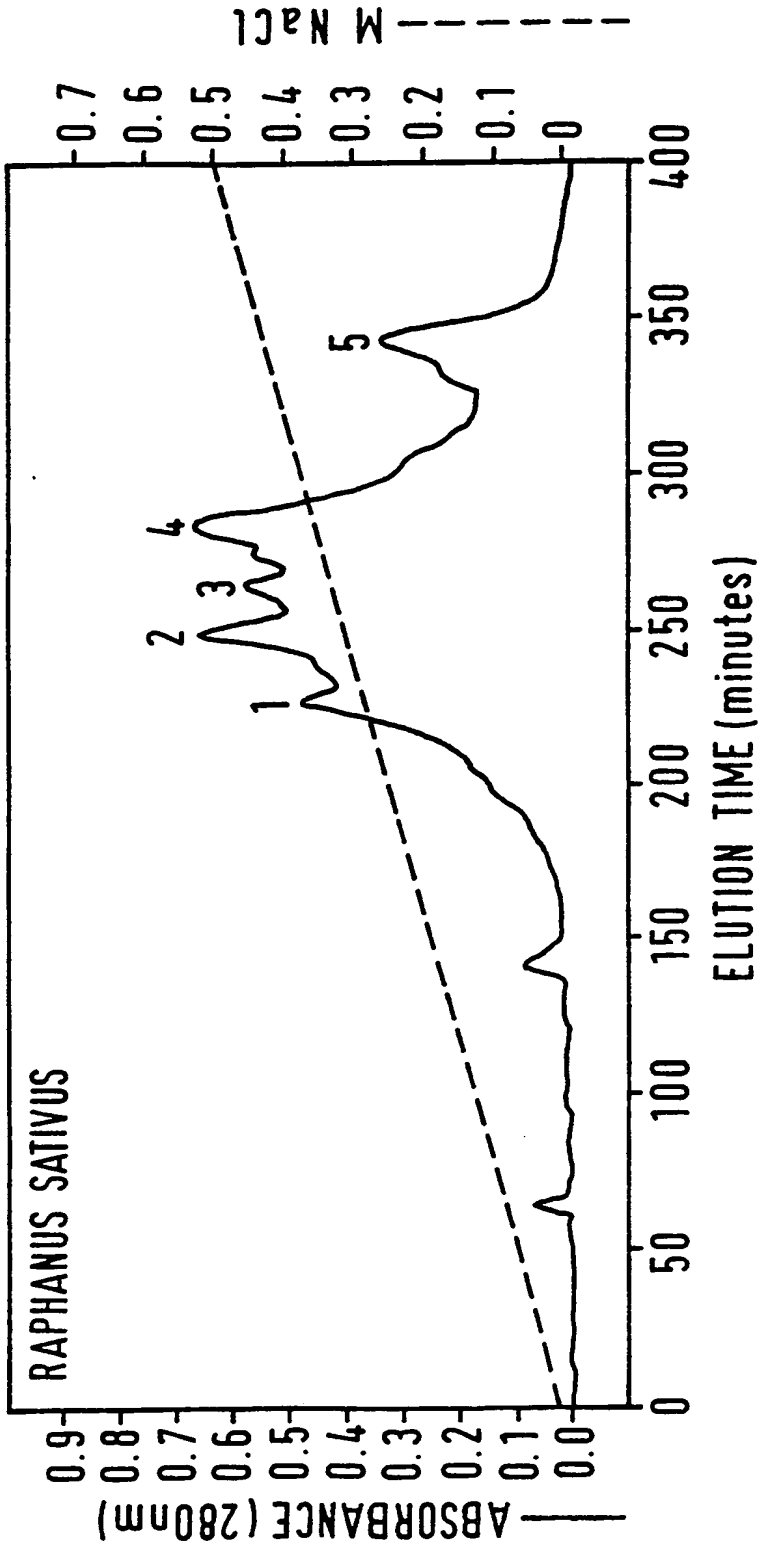


FIG. 2

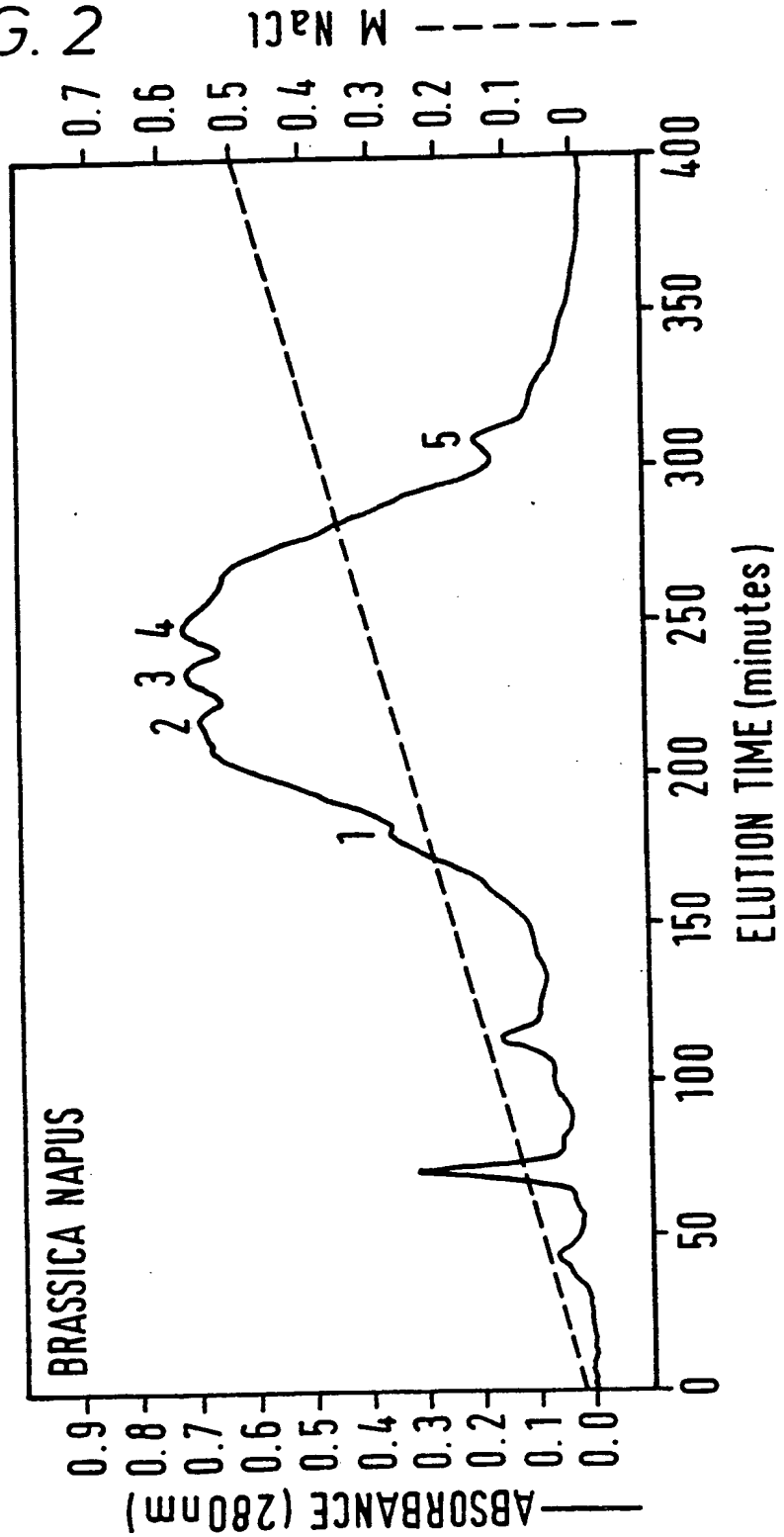

M NaCl
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
400
350
300
250
200
150
100
50
0
ELUTION TIME (minutes)
1
2
3
4
5
BRASSICA NAPUS
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE (280nm)

FIG. 3

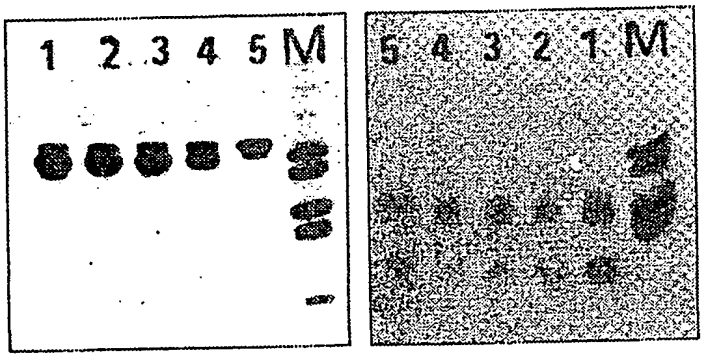
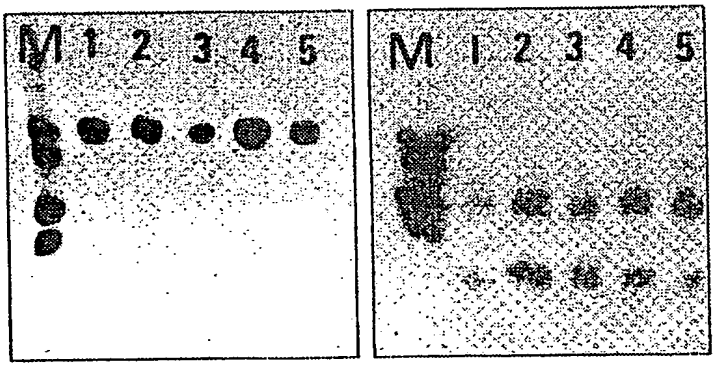


FIG. 4



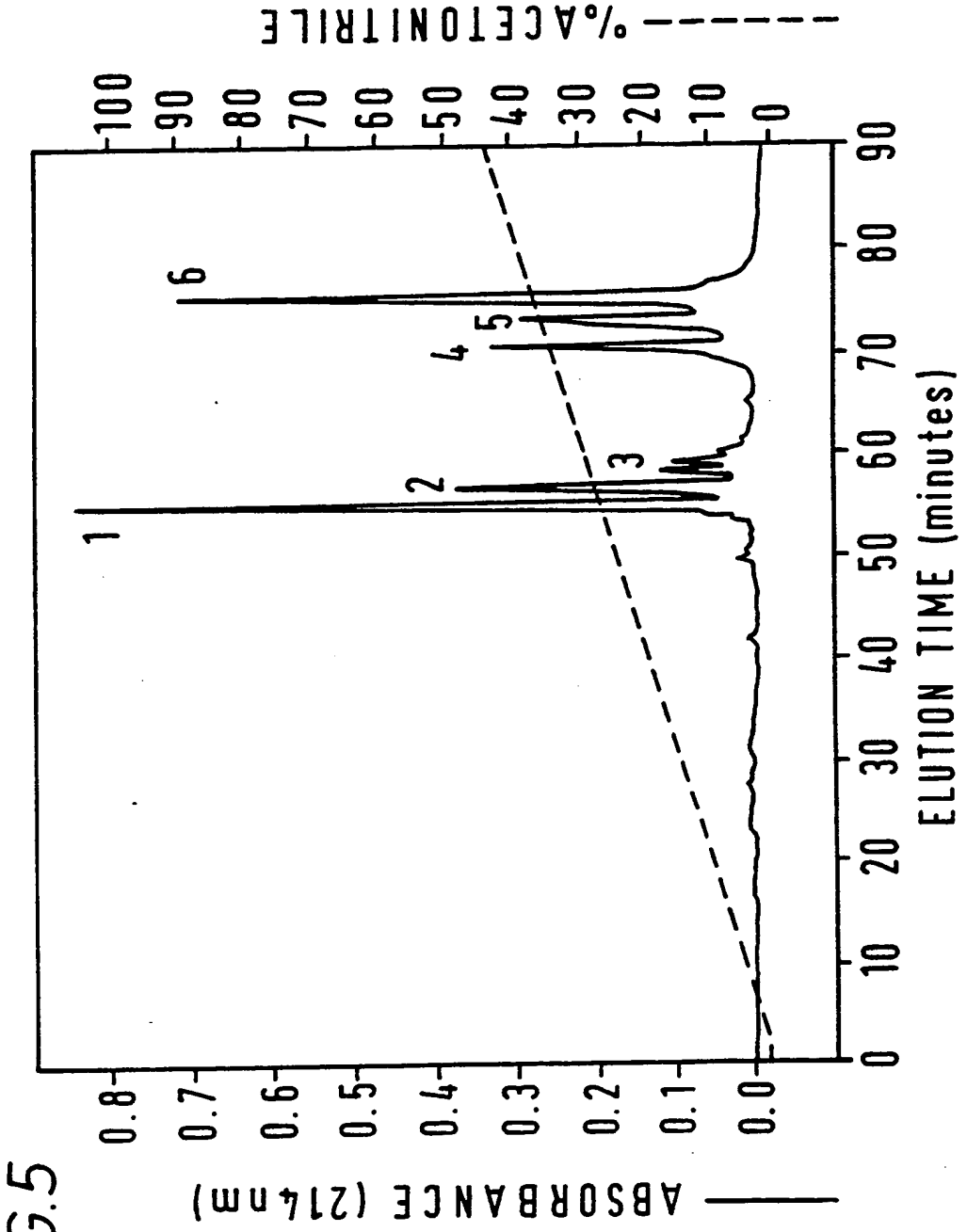

ABSORBANCE (214nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ELUTION TIME (minutes)
0
10
20
30
40
50
60
70
80
90
%ACETONITRILE
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6

FIG.5

FIG.6

Small subunit

Rs-2S5	PAGPFRIPRCRREFQQAQHLRACQQWLHRQ
pBa3	PAGPFRIPRCRREFQQAQHLRACQQWLHRQ
Napin	sAGPFRIPkCRkEFQQAQHLRACQQWLHkQ

Large subunit

Rs-2S5	PQGPQQRPPLLQQCCNNLLQ
pBA3	PQGPQQRPPLLQQCCNeLkQ
Napin	PQGPQQRPPLLQQCCNeLkQ

FIG.7

bti0	FGDSCAPGDAL
bti1	AGKKRPWKCC
bti2	AGKKIPWKCC
WG11	AAKKRPWKCC

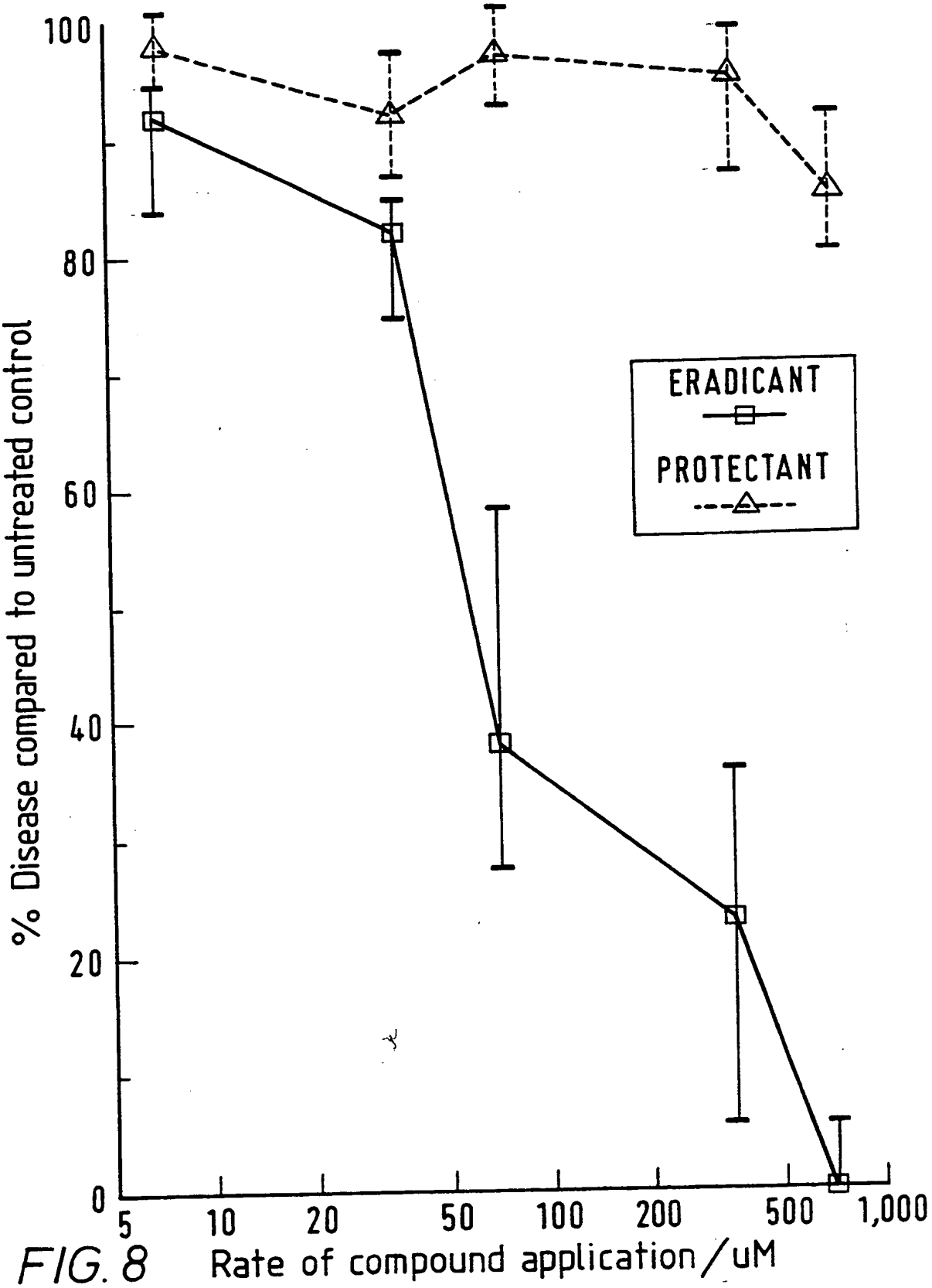

100
80
60
40
20
0
% Disease compared to untreated control
ERADICANT
PROTECTANT
5
10
20
50
100
200
500
1,000
Rate of compound application / uM

FIG. 8

FIG. 9

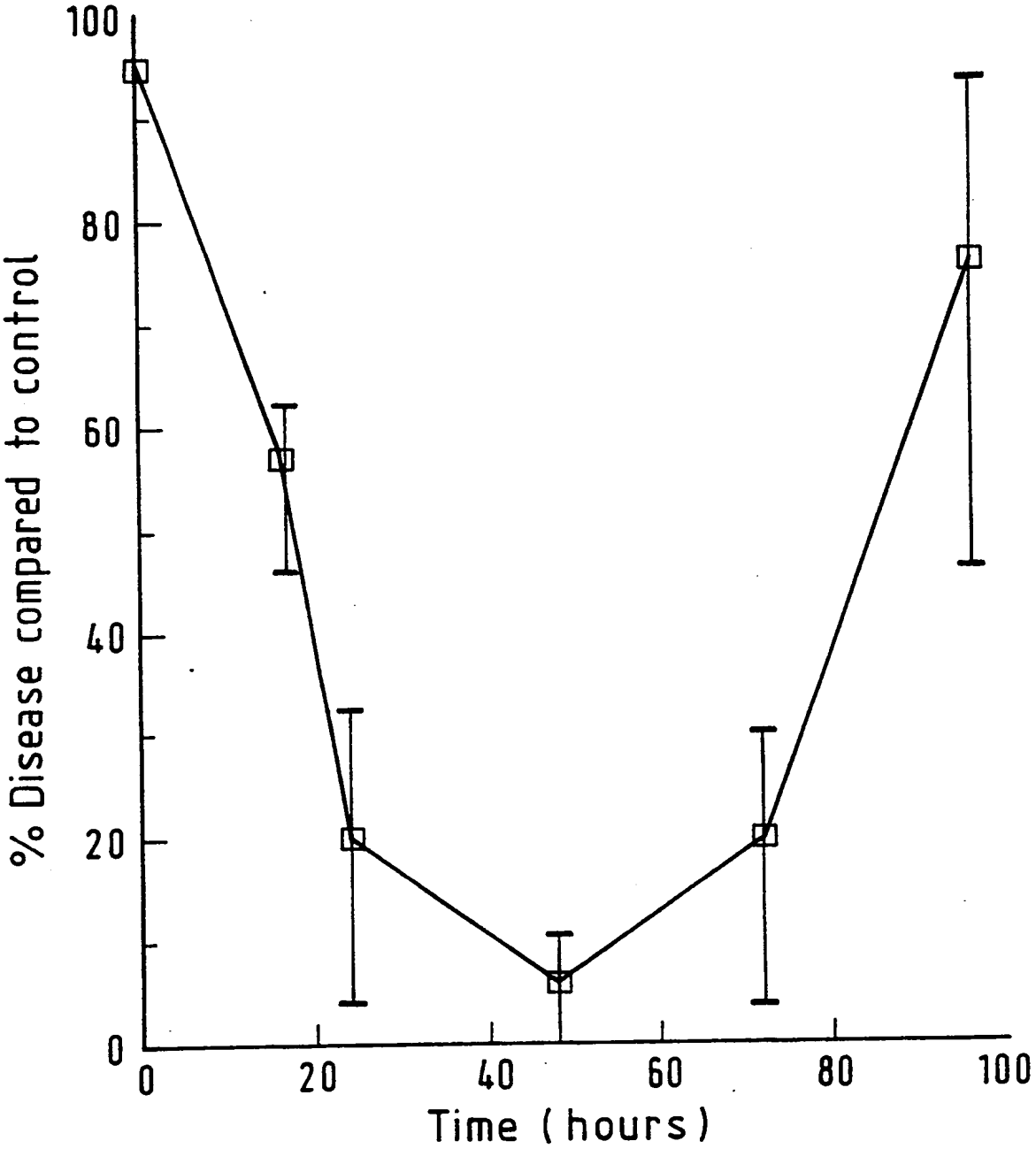


FIG. 10 (1/2)

```
          10        20        30        40        50        60        70
          |         |         |         |         |         |         |
GGATCCCATACACGAATGGCGAACAAGCTCTTCCTCGTCTCGGCAACTCTCGCCTTCTTCTTCCTTCTCACC
          M  A  N  K  L  F  L  V  S  A  T  L  A  F  F  F  L  L  T

          80        90        100       110       120       130       140
          |         |         |         |         |         |         |
AACGCCTCCATCTACAGGACGGTCGTCGAGTTCGACGAAGATGATGCCACGAACCCAGCCGGCCCATTTAGG
 N  A  S  I  Y  R  T  V  V  E  F  D  E  D  D  A  T  N  P  A  G  P  F  R

          150       160       170       180       190       200       210
          |         |         |         |         |         |         |
ATTCCAAGATGCAGGAAGGAGTTTCAGCAAGCACAACACCTGAGAGCTTGCCAGCAATGGCTCCACAAGCAA
 I  P  R  C  R  K  E  F  Q  Q  A  Q  H  L  R  A  C  Q  Q  W  L  H  K  Q

          220       230       240       250       260       270       280
          |         |         |         |         |         |         |
GCAATGCAGGTCCGTGGTGGTCCTAGCTTGGCCCTCGATGGTGAGTTTGACTTTGAAGATGACATGGAGAAC
 A  M  Q  V  R  G  G  P  S  L  A  L  D  G  E  F  D  F  E  D  D  M  E  N
```

FIG. 10 (2/2)

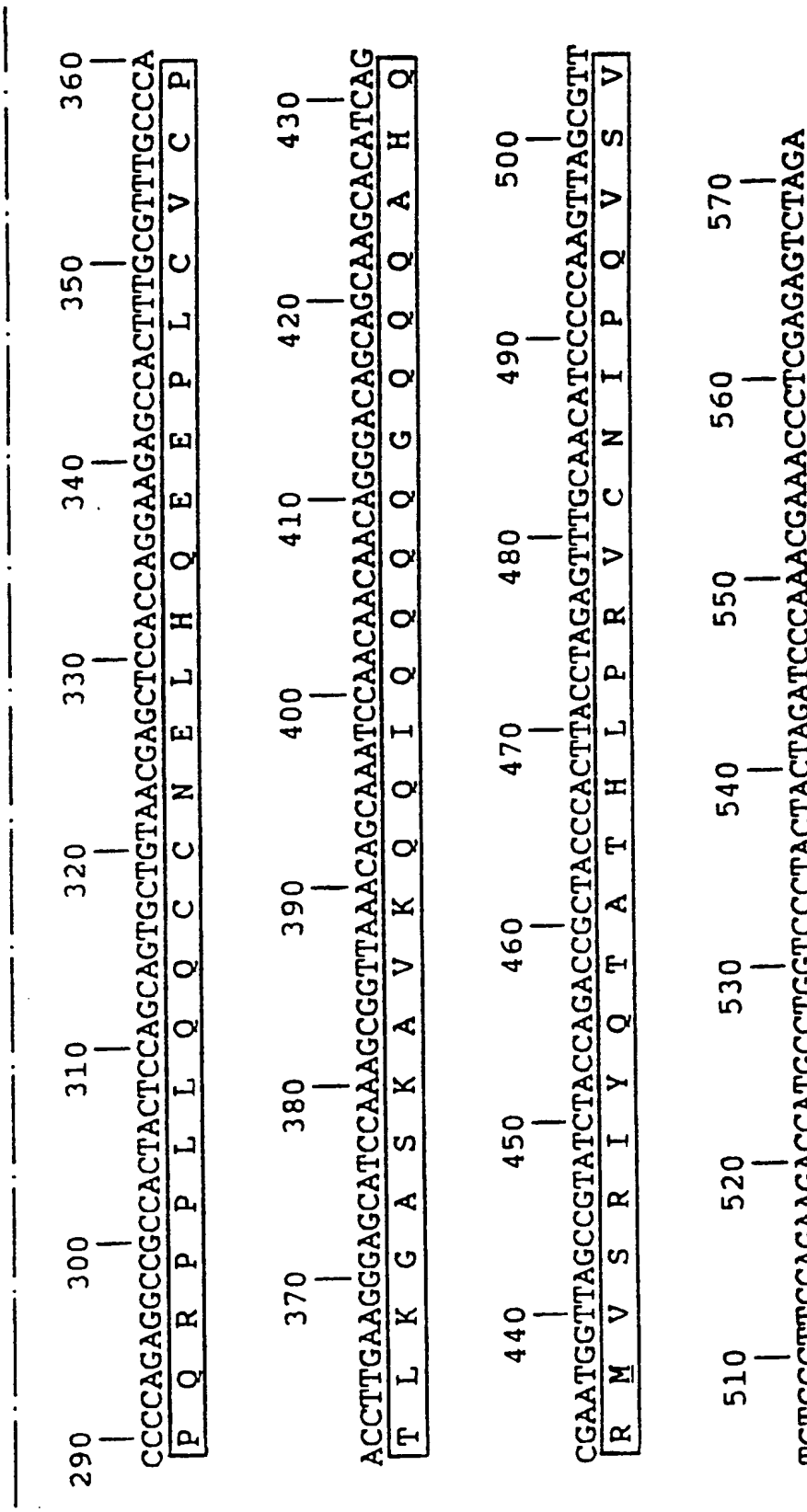

290 300 310 320 330 340 350 360
CCCCAGAGGCCGCCACTACTCCAGCAGTGCTGTAACGAGCTCCACCAGGAAGAGCCACTTGCGTTTGCCCA
P Q R P P L L Q Q C C N E L H Q E E P L C V C P
370 380 390 400 410 420 430
ACCTTGAAGGGAGCATCCAAAGCGGTTAAACAGCAAATCCAACAACAACAGGGACAGCAGCAAGCACATCAG
T L K G A S K A V K Q Q I Q Q Q Q Q G Q Q Q A H Q
440 450 460 470 480 490 500
CGAATGGTTAGCCGTATCTACCAGACCGCTACCCACTTACCTAGAGTTTGCAACATCCCCCAAGTTAGCGTT
R M V S R I Y Q T A T H L P R V C N I P Q V S V
510 520 530 540 550 560 570
TGTCCCTTCCAGAAGACCATGCCTGGTCCCTACTACTAGATCCCAAACGAAACCCTCGAGAGTCTAGA
C P F Q K T M P G P Y Y -

FIG. 11

```
          10         20         30         40         50         60         70
          |          |          |          |          |          |          |
GAATTCGAGCTCGGTACCCATCATTCAAATATACTAAACTAATTATAAAAAATGGCTAAGGTTCCAATTGCC
                                                      M  A  K  V  P  I  A

          80         90         100        110        120        130        140
          |          |          |          |          |          |          |
TTTCTCAAATTCGTCATCGTGTTGATTCTCTTCATTGCCATGTCAGGCATGATAGAAGCTAGCGCCGGCCCA
 F  L  K  F  V  I  V  L  I  L  F  I  A  M  S  G  M  I  E  A  S  A  G  P

          150        160        170        180        190        200        210
          |          |          |          |          |          |          |
TTCAGGATTCCAAGATGTAGGAGGGAGTTTCAGCAAGCACAACACCTAAGAGCTTGCCAACAATGGCTCCAC
 F  R  I  P  R  C  R  R  E  F  Q  Q  A  Q  H  L  R  A  C  Q  Q  W  L  H

          220        230        240        250        260
          |          |          |          |          |
AGGCAGGCAAGGCAGTCCGGTTAGTCTAGCTATAGATCTAGTCGAC
 R  Q  A  R  Q  S  G  -
```

FIG. 12

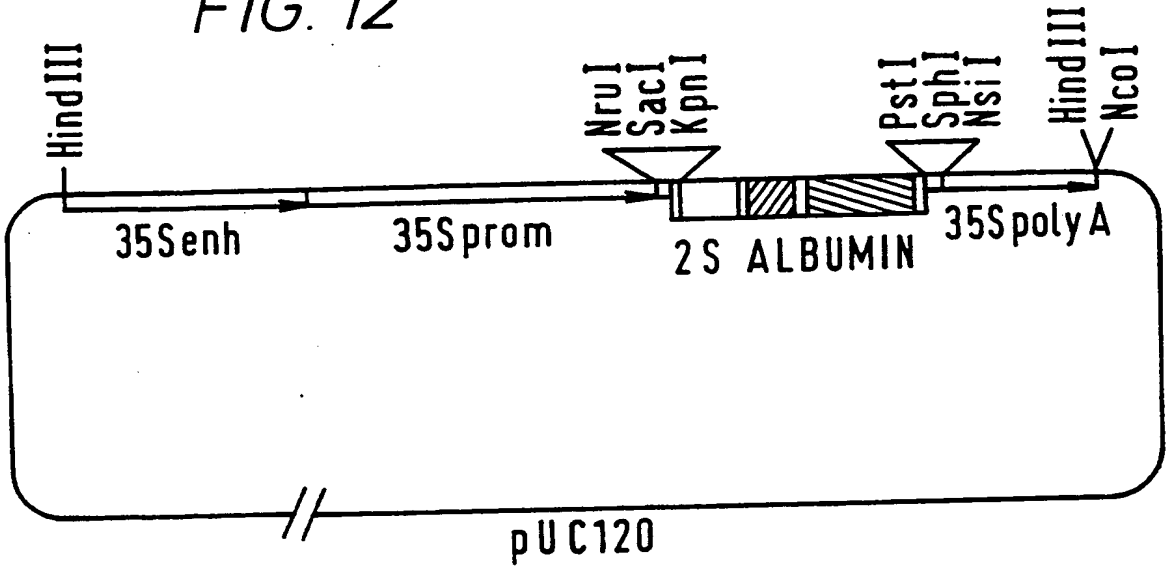

HindIII
NruI
SacI
KpnI
PstI
SphI
NsiI
HindIII
NcoI
35Senh
35Sprom
2S ALBUMIN
35SpolyA
pUC120

FIG. 13

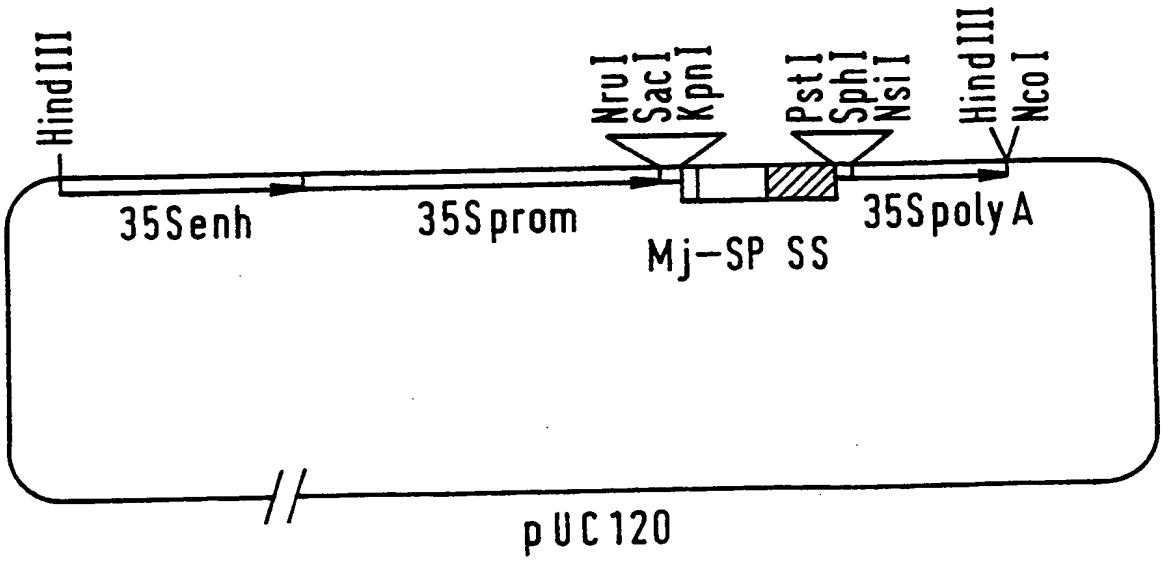

HindIII
NruI
SacI
KpnI
PstI
SphI
NsiI
HindIII
NcoI
35Senh
35Sprom
Mj-SP SS
35SpolyA
pUC120

FIG. 14

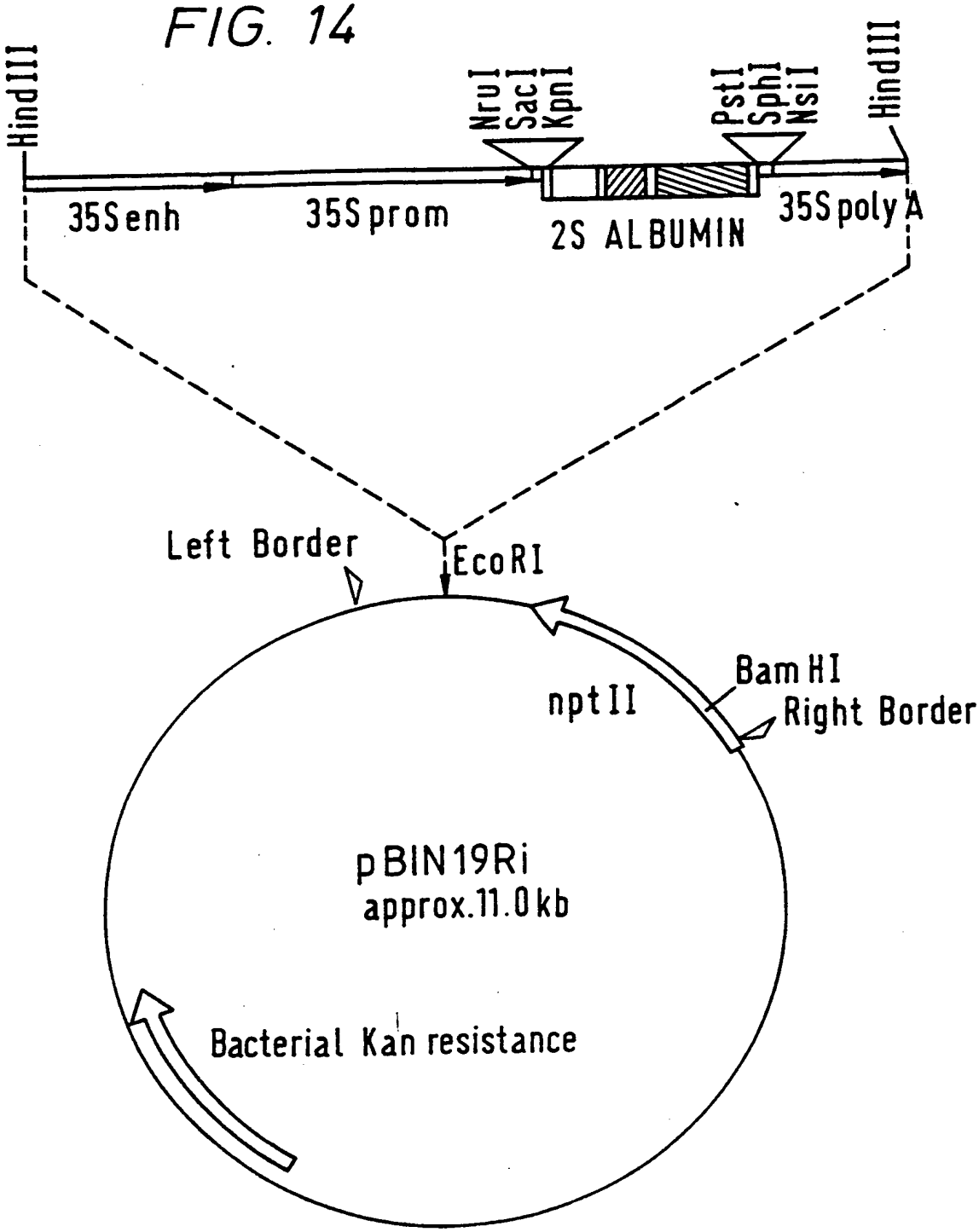
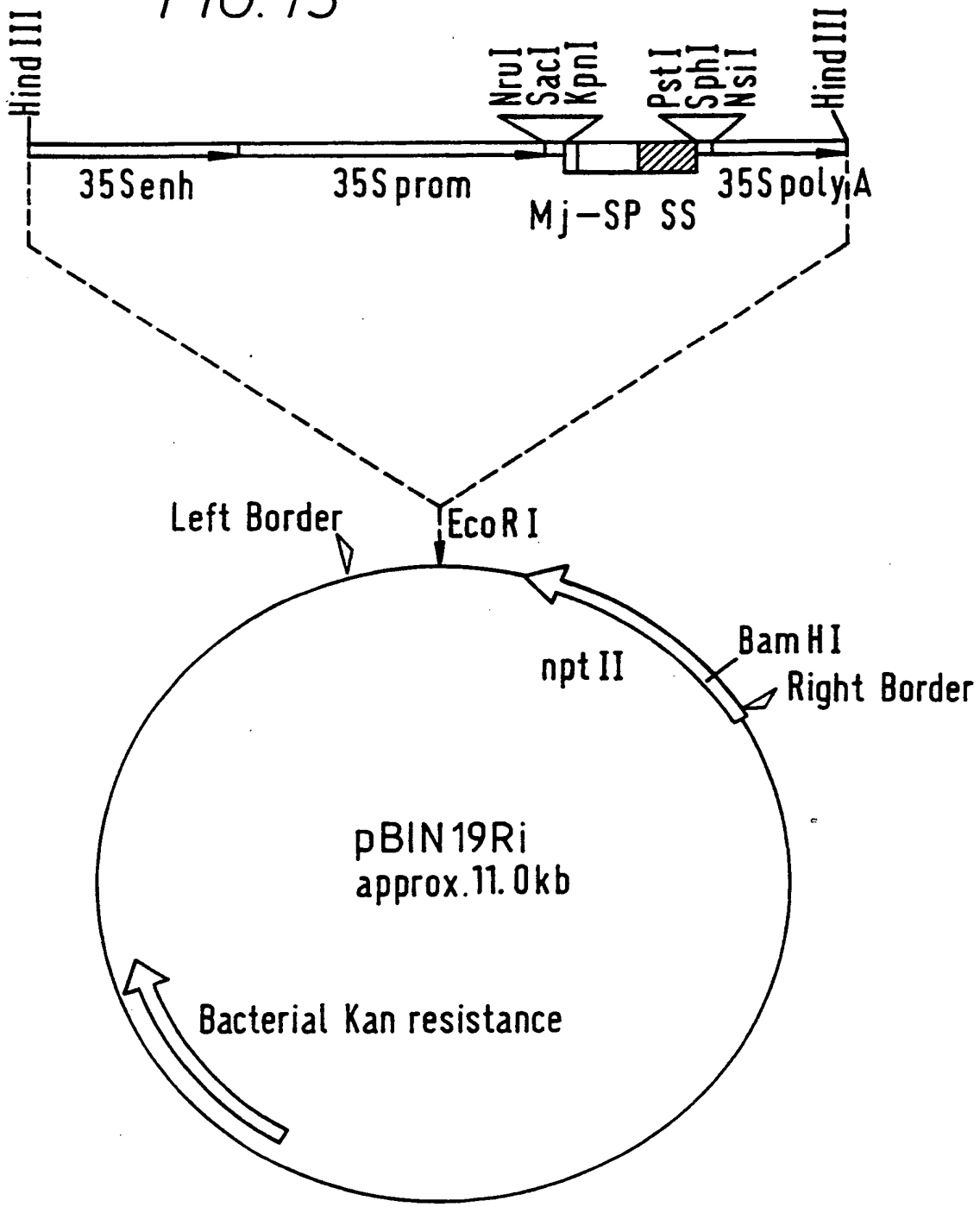

HindIII
NruI
SacI
KpnI
PstI
SphI
NsiI
HindIII
35S enh
35S prom
2S ALBUMIN
35S poly A
Left Border
EcoRI
Bam HI
Right Border
npt II
pBIN19Ri
approx.11.0kb
Bacterial Kan resistance

FIG. 15


Hind III
NruI
SacI
KpnI
PstI
SphI
NsiI
Hind III
35S enh
35S prom
Mj-SP SS
35S poly A
Left Border
EcoR I
Bam H I
npt II
Right Border
pBIN19Ri
approx. 11.0kb
Bacterial Kan resistance

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01574

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 01 N 63/00, 65/00; C 07 K 15/10; C 12 N 15/29; A 01 H 5/00, 5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 01 N; C 07 K, C 12 N; A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	Journal of Biological Chemistry, vol. 267, No. 22, August 1992, F.R.G. Terras et al.: "Analysis of Two Novel Classes of Plant Antifungal Proteins from Radish (Raphanus sativus L.) Seeds ", pp. 15301-15309, see the whole document --	1-21
P,X	Bot. Bull. Academia Sinica, vol. 33, No. 2, April 1992, R.H. Sammour et al.: "Antimicrobial activity of legume seed proteins ", pp. 185-190, see the whole document --	1-21
X	EP, A1, 0352010 (HUMBER GROWERS MARKETING ORGANISATION LIMITED) 24 January 1990, see page 3, line 11; page 4, lines 31-35, lines 56-57; claims 1, 8, 9 --	1-10

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th November 1992	01 DEC 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Gerd Wranne

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	EP, A1, 0375091 (INSTITUT FUR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH) 27 June 1990, see column 18, lines 25-47, example 6; claims 14-28	10-21
X	Patent Abstracts of Japan, Vol 7, No 114, C166, abstract of JP 58- 32808, publ 1983-02-25 KUREHA KAGAKU KOGYO K.K. et al.	1-10
X	EP, A1, 0135343 (AGRICULTURAL GENETICS COMPANY LIMITED) 27 March 1985, see the claims	1-10
X	EP, A2, 0348348 (CIBA-GEIGY AG) 27 December 1989, see claims 1-12, 37-39, 63-72	1-21
P,X	WO, A1, 9118512 (WASHINGTON STATE UNIVERSITY RESEARCH FOUNDATION) 12 December 1991, see page 5; page 11, last paragraph - page 12, first paragraph	1-10
A	PHYSIOLOGIA PLANTARUM, vol. 80, 1990, Klaus Apel et al.: "Leaf thionins, a novel class of putative defence factors ", pp. 315-321, see the whole document	1-21
A	MOLECULAR PLANT-MICROBE INTERACTIONS, vol. 4, No. 4, 1991, Alison J. Vigers et al.: "A New Family of Plant Antifungal Proteins ", pp. 315-323, see the whole document	1-10

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 92/01574

SA 63943

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0352010	24/01/90	AU-D-	3983589	05/02/90
		EP-A-	0424454	02/05/91
		GB-A-B-	2239602	10/07/91
		JP-T-	3505876	19/12/91
		WO-A-	90/00351	25/01/90
EP-A1- 0375091	27/06/90	DE-A-	3843628	05/07/90
		JP-A-	2283275	20/11/90
EP-A1- 0135343	27/03/85	US-A-	4640836	03/02/87
EP-A2- 0348348	27/12/89	AU-D-	3656889	21/12/89
		JP-A-	2046238	15/02/90
WO-A1- 9118512	12/12/91	AU-D-	7953191	31/12/91

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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